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(57) Abstract

The invention detects the presence of telomerase activity by employing a test nucleic acid sequence that is employed as a substrate by telomerase for addition of telomeric repeat sequence. The detection of the elongation sequences indicate the presence of telomerase activity. In addition, the methods of the invention can be utilized to determine the inhibitory or enhancing effect of a compound on telomerase activity.

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TELOMERASE EXTENSION ASSAY

Background of the Invention

Telomeres are tandem repeats at the ends of eukaryotic chromosomes that appear to function in chromosome replication. Telomerase is a ribonucleoprotein enzyme that synthesizes one strand of the telomeric DNA using as a template a sequence contained within the RNA component of the enzyme. See. e.g., Blackburn (1992) *Annu. Rev. Biochem.* 61:113-129, incorporated herein by reference. Human telomerase is known to synthesize telomeric repeat units with the sequence 5'-TTAGGG-3'. See. e.g., Morin (1989) *Cell.* 59:521-529. and Morin (1991) *Nature* 353:454-456. incorporated herein by reference.

The presence of DNA telomerase has been correlated with unscheduled DNA replication events such as those that occur in various cancers. For example, telomere shortening appears to dictate the number of cell divisions a normal somatic cell can undergo. Telomerase activity has also been linked to senescence. Whereas normal cells with relatively long telomeres and a senescent phenotype may contain little or no telomerase activity, tumor cells with short telomeres may have significant telomerase activity. Thus. organismal senescence might be halted or at least slowed if an effective. non-toxic telomerase inhibitor can be found. Accordingly, the identification of compounds that affect telomerase activity provides important benefits to efforts at treating human diseases such as cancer.

Assays for the detection of cells which express telomerase have been reported (see, e.g., U.S. Patent No. 5,583,016 to Villeponteau et al.). Recently, assays which can measure telomerase levels in biopsy specimens have been reported (see. e.g., Sugino *et al.* (1996) *Int. J. Cancer* 69:301-6; Kim *et al.* (1994) *Science* 266: 2011-5). However, such assays are not always convenient or rapid. and can depend upon PCR amplification of nucleic acid samples, which could result in errors. Accordingly, this invention provides methods for detection of telomerase that are easy to use and accurate.

Summary of the Invention

The present invention relates to methods of measuring levels of telomerase, e.g., human telomerase activity, in a variety of samples, e.g., a biological sample. The

invention also includes methods for screening compounds for their ability to modulate, e.g., to enhance or inhibit, telomerase activity, e.g., as a high throughput drug screening assay for pharmaceuticals which affect telomerase activity.

In one aspect, the invention provides a method for screening for compounds that modulate telomerase activity. In particular, the method can detect telomerase activity by incubating a sample that is suspected of having telomerase activity with a nucleic acid test sequence and detecting elongation of the test sequence by telomerase. The comparison of the level of telomerase activity in the absence of a test compound with the level of activity in its presence can provide information regarding the inhibitory or the enhancing effects of the compound. For example, a compound shows inhibitory effects if the elongation occurs in its absence, but fails in its presence.

The preferred embodiments of the invention perform the test for telomerase activity *in vitro* by employing test nucleic acid sequences, preferably DNA sequences, that are other than a preparation of genomic DNA or cDNA library. These embodiments utilize a substantially pure nucleic acid sequence that is bound to a solid substrate and is exogenously added to a sample whose telomerase activity is being investigated.

One aspect of the present assay relates to employing a hairpin nucleic acid sequence, having a single stranded region with a 3'-terminus, that is affixed to a solid substrate. Telomerase in presence of appropriate oligonucleotides can add telomeric repeat sequences to the 3'-terminus of the hairpin.

According to another aspect of the invention, the elongation of the test nucleic acid sequence is detected by adding a detector sequence that is selected to be complementary to a portion of the test sequence that includes at least one telomeric repeat sequence, thus hybridizing to this portion. The assay detects the hybridized detector sequence by providing a label, e.g., a radiolabel, in the detector sequence or adding such a label to the detector sequence. For example, addition of reagents that can hybridize to a section of the detector sequence and cause chemiluminescence or color change of the sample results in detection of telomerase activity.

These and other aspects of the invention are described in more detail below.

Brief Description of the Drawing

FIGURE 1 is a schematic depiction of the various steps of a preferred embodiment of the telomerase assay of the invention.

Detailed Description of the Invention

The invention relates to methods for detecting telomerase activity and for screening for compounds that modulate the activity of telomerase *in vivo* or *in vitro*. The methods of the invention can identify compounds which may be useful for the treatment of conditions related to activity of telomerase. The invention also relates to methods for detecting the presence or absence of telomerase activity in a sample such as a biological sample, a tissue biopsy sample or a biological fluid, e.g., blood, urine, saliva, and the like. The telomerase assays of the invention are useful for detecting tissue (or other biological materials) which has telomerase activity, which may be an indicator of telomerase-linked conditions such as the presence of cancerous or precancerous cells. The invention thus provides methods for rapidly screening biological samples for conditions such as cancer.

In one embodiment, the invention provides a method for detecting telomerase activity in a sample. The method includes the steps of contacting a test nucleic acid sequence with a sample suspected of containing telomerase activity, under conditions such that if telomerase is present in the sample, the nucleic acid sequence is elongated by telomerase to form an elongated nucleic acid sequence, and detecting the elongated nucleic acid sequence. The sample can include cells, which can be lysed if desired, under conditions such that telomerase is not denatured, i.e., such that telomerase activity is not destroyed.

In another embodiment, the invention provides a method for screening for compounds capable of modulating telomerase activity. The method includes the steps of contacting a nucleic acid sequence with a sample suspected of containing a telomerase, in the presence of a test compound, under conditions such that if telomerase is present in the sample, the nucleic acid sequence is elongated by telomerase to form an elongated nucleic acid sequence in the absence of the test compound, and detecting the presence or absence of the elongated nucleic acid sequence. Compounds which suppress the activity of telomerase may be useful as inhibitors of telomerase activity *in vitro*, and may

therefore be useful as inhibitors of carcinogenesis or cell senescence, as described above. Compounds which enhance the activity of telomerase in the assays of the invention may be promoters of cancer or cell senescence. Accordingly, the assays of the invention provide the ability to screen for both potentially harmful and potentially therapeutic compounds.

In preferred embodiments, methods of the invention typically employ a nucleic acid hairpin having a 3'-terminus to which telomerase can add telomeric repeat sequences. In a particularly preferred embodiment, the nucleic acid sequence (e.g., a hairpin; see W097/08183 and U.S. Patent Application No. 08/519.197) includes at least one telomeric repeat sequence as a terminal portion of the hairpin sequence (e.g., TTAGGG-3'). Telomeric repeat sequences include the human telomeric repeat sequence (5'-TTAGGG-3'), and other telomeric repeat sequences known to one of ordinary skill in the art.

FIGURE 1 shows schematically that the nucleic acid test sequence can be immobilized on a solid support. In the illustrative embodiment of FIGURE 1, the test sequence is a nucleic acid "hairpin", e.g., a nucleic acid molecule having self-complementary regions capable of hybridizing to each other to form a duplex "stem" region and, preferably, at least one dangling single-stranded portion. The advantages of immobilization of the test sequence include ease of handling, and manipulation, e.g., washing, separating, etc. The immobilization is achieved by a variety of methods known in the art. One embodiment incorporates commercially available biotinylated nucleic acid bases into the test sequence in order to attach the test sequence to a solid support that has been derivatized with avidin or streptavidin, e.g., streptavidin-coated 96-well plates, or streptavidin-coated beads such as Dynabeads, available for Dynal. Other methods for attaching or immobilizing a nucleic acid sequence on a solid support will be apparent to one of ordinary skill in the art.

In a preferred embodiment, a nucleic acid hairpin, which has been affixed to a solid support, is contacted with a sample that is suspected of having telomerase activity. As described above, telomerase is known to elongate a strand of nucleic acid by addition of telomeric repeat sequences. According to the methods of the invention, presence of telomerase activity in the sample is detected by determining whether tandemly repeated telomeric sequences have been added to the test hairpin strand. The elongation process requires the presence of appropriate oligonucleotide phosphates ("NTP"), i.e., NTPs

required for synthesis of a telomeric repeat sequence. Such NTPs can be readily purchased from commercial suppliers and are added to the reaction mixture to permit the telomerase extension reaction to proceed (e.g., human telomerase requires ATP, TTP, and GTP). Accordingly, if telomerase is present in the sample, it recognizes the 3'-terminus of the test sequence as a substrate and extends it by addition of telomeric repeat sequences. In certain embodiments, the test sequence includes a telomeric repeat sequence before addition of a sample which contains telomerase, e.g., a synthetic test sequence which is provided with a telomeric repeat sequence at the 3'-terminus by chemical synthesis. A preferred 3'-terminal portion of the test sequence is 5'-CTGGGTTAGGG-3'. In one particularly preferred embodiment, the test nucleic acid sequence has the sequence 5'-CTAGT CGACG TGGTC CTTT(biotin)T T TGGAC CACGT CGACT AGCTG GGTTA GGG-3' , where the T(biotin) denotes a biotinylated thymidine.

The methods of the invention (e.g., as described in Example 1, infra) can detect the presence of elongated nucleic acids with sufficient sensitivity such that no target replication steps such as Polymerase Chain Reaction ("PCR") or Ligase Chain Reaction ("LCR") are required, and in a preferred embodiment no PCR or LCR steps are performed. Application of such replication methods typically involve various steps including selection of appropriate primers and necessary enzymes, heating and cooling cycles and the like. The elimination of such steps by the preferred embodiments can render the methods of the invention more convenient to use and less costly in comparison with those that utilize replication procedures. However, in certain embodiments it can be advantageous to replicate the elongated nucleic acid (or its complement) or a portion thereof, e.g., by PCR, LCR, or other known methods. For example, one may desire to incorporate such methods in the present invention if ultrasensivity of detection of telomerase is required.

The methods of the invention detect repeat sequences added by telomerase to the nucleic acid substrate (e.g., a hairpin) by employing detection sequences that are complementary to a portion of the single-stranded region of the hairpin that includes at least one repeat sequence. The provision of a detector sequence which is complementary to a region of the hairpin as well as to at least a portion of a telomeric repeat sequence rather than to a single strand provides certain advantages. In particular, upon hybridization of the hairpin with a suitably selected detector sequence, a "nicked" duplex structure is formed, comprising contiguous regions of intramolecular hairpin:hairpin

duplex and intermolecular detector:hairpin duplex. This arrangement provides base stacking between the intramolecular duplex (i.e., the duplex "stem" of the hairpin) and the intermolecular duplex (i.e., the detector:hairpin duplex), which results in a greater sequence stringency than hybridization to a simple single strand. Further, the duplex region of the hairpin stabilizes, e.g., entropically, the detector-specific region of the hairpin and thereby favors formation of a detector:hairpin duplex.

The length of the single stranded region of the hairpin can be selected to allow the use of a variety of detector sequences. In particular, if detection of only a few telomeric repeat sequences is desired, it may be advantageous to select a hairpin with a relatively long single stranded region to allow stable attachment of a detector sequence that has only a few sequences that are complementary to telomeric repeat sequences.

In one embodiment, the detector sequence preferably contains at least a portion which is complementary to at least two telomeric repeat sequences, e.g., the sequence 5'-CCCTAACCCTAA-3' or 5'- CCCUAACCCUAA-3'. The detector sequence also can include a poly(T), e.g., T_{30}, "tail" region that is not complementary to the hairpin. In this example, if the single stranded region of the hairpin contains the sequence 5'-TTAGGGTTAGGG-3', i.e., if the hairpin has been elongated by telomerase, the detector sequence hybridizes to this segment. Excess detector sequences and non-specifically-bound detector sequences are removed by washing with a buffer having an appropriate stringency to remove unbound detector sequences while not removing specifically-bound detector molecules, as known in the art.

The hybridized detector sequences are then detected by well-known methods in the art for detecting nucleic acid sequences. In some embodiments of the present invention the detector sequence contains a label, e.g., radiolabel, that allows a direct detection of the sequence. In other embodiments, the sequence is detected indirectly by attaching a label to a portion of the detector sequence which can be subsequently detected by direct or indirect means. Labels can include radioisotopes, fluorophores, chemiluminescent tags and the like.

For example, European Patent Publication EP 128 332, herein incorporated by reference, reports one such method that employs a "bridging moiety" which provides a bridge between an analyte and a "signaling moiety" which provides a detectable signal. The "signal generating" portion of the signaling entity can encompass virtually any of

the signal generating systems used in the prior art. In particular, it comprises a moiety which generates a signal itself, e.g., a radiolabel, or a moiety which , upon further reaction or manipulation, will give rise to a signal, e.g., an enzyme-linked system. Some other methods utilize radioactive nucleotides in a label and detect a nucleotide sequence to which such a label has hybridized by detecting the decay of the radioactive nucleotides.

A preferred embodiment of the present invention employs a detector sequence shown in FIGURE 1 that is selected to have a poly(T) or poly(dT) "tail" to which a complementary probe sequence, i.e., poly $d(A)_{1500}$, hybridizes. The probe sequence is chosen to be long enough so as to allow attachment of various labels in the regions that are not hybridized to the detector sequence. In particular, after removing excess polyd(A), i.e., unhybridized polyd(A), a labeled reagent such a $T_{20}FITC_2$ (T_{20} = a sequence of poly (T) having 20 T units; FITC = fluoreseinisothiocycanate) which can be synthesized according to known procedures, is added to the reaction mixture and it hybridizes with a portion of the polyd(A) region. After the labeling reagent is added, addition of anti-FITC-AP (conjugate of anti-FITC antibody (commercially available) with alkaline phosphatase (AP)), which specifically binds to FITC, followed by appropriate washing steps to remove excess anti-FITC-AP, provides a detectable moiety for indicating the presence of telomerase activity in the sample, e.g., by addition of a color-forming substrate for AP, followed by spectrophotometric detection of any color formation.

Example 1 provides further clarification of various steps of a preferred embodiment of the invention and is meant as illustrative and not in a limiting sense.

Example 1

In this example, a synthetic nucleic acid "hairpin" is used as a substrate for elongation by telomerase. Appropriate nucleic acid hairpins can be designed and prepared by the skilled artisan (see, e.g., U.S. Patent Application Serial No. 08/519,197, filed August 25, 1995, which is hereby incorporated by reference). Referring to Figure 1, the synthetic hairpin is first affixed to a solid support in a reaction vessel (such as a 96-well plate), e.g., through a biotin-streptavidin linkage or other means known in the art. A sample to be tested (which may contain telomerase) is then incubated with the solid-supported hairpin. Telomerase present in the sample (if any) extends the dangling

single strand of the hairpin at the 3'-terminus, attaching six base repeats of the sequence 5'-TTAGGG-3'. After the sample has been incubated in contact with the immobilized hairpins for a pre-selected period of time, the bound hairpins are (optionally) washed to remove sample, and a "detector" sequence is supplied to the reaction vessel. The detector sequence includes at least one sequence which is complimentary to the telomerase extension products, i.e., the detector hybridizes to the bound hairpin only if two or more telomerase generated hexamer sequences (5'-TTAGGG-3') are present. The detector sequence preferably includes the sequence 5'-CCCTAACCCTAA-3' (or 5'-CCCUAACCCUAA-3'). In this example, the detector further include a poly(T) (e.g., T_{30}) "tail", i.e., a portion which is not complementary to the hairpin. If the bound hairpins contain the sequence 5'-TTAGGGTTAGGG-3' (i.e., have been extended by telomerase), the detector sequence (or a portion thereof) will hybridize and bind to the hairpin. Excess detector, and non-specifically-bound detector sequence, can be removed by washing with buffer of the appropriate stringency, as is known in the art.

A probe complementary to the poly(T) "tail" of the detector sequence, e.g., polyd $(A)_{1500}$, is added, and hybridizes to the T_{30} tail of the "detector" under appropriate conditions. Certain polyd(A) (or poly(A) sequences are commercially available (e.g., from Sigma Chemical Co., St. Louis, MO) or can be prepared by methods known in the art. Excess polyd(A) can be removed by washing, followed by addition of a labeled reagent such as T_{20} $FITC_2$, (T_{20} = a sequence of poly (T) having 20 T units; FITC = fluoresceinisothiocycanate; the T_{20} $FITC_2$ reagent can be synthesized according to known procedures). After the labeling reagent is added, addition of anti-FITC-AP (conjugate of anti-FITC antibody (commercially available) with alkaline phosphatase (AP)), which specifically binds to FITC, followed by appropriate washing steps to remove excess anti-FITC-AP, provides a detectable moiety for indicating the presence of telomerase activity in the sample (e.g., by addition of a color-forming substrate for AP, followed by spectrophotometric detection of any color formation). The complex of hairpin (which, in Figure 1, has been extended by telomerase present in the sample), detector, polyd(A), T_{20} $FITC_2$, and anti-FITC-AP is depicted in Figure 1 as the "Telomerase Extended Hairpin: Detector: Amplifier complex".

Addition of the substrate for AP results in a visible color or chemiluminescent signal, which can be read by standard methods, e.g., by spectrophotometric detection with an automated plate reader

Thus, the methods of the invention attain the above-mentioned objectives including detection of telomerase activity.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation. numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

The contents of all references and patent applications cited herein are hereby incorporated by reference.

Other embodiments are within the following claims.

What is claimed is:

1. A method for detecting telomerase activity in a sample, comprising the steps of contacting a nucleic acid sequence with a sample suspected of containing a telomerase, under conditions such that if telomerase is present in the sample, the nucleic acid sequence is elongated by telomerase to form an elongated nucleic acid sequence, and

detecting the elongated nucleic acid sequence.

2. The method of claim 1, wherein the nucleic acid sequence is immobilized on a solid support.

3. The method of claim 1, wherein the nucleic acid sequence is a nucleic acid hairpin

4. The method of claim 1, wherein the nucleic acid sequence comprises a telomeric repeat sequence prior to elongation by telomerase.

5. A method for screening for compounds capable of modulating telomerase activity, comprising the steps of

contacting a nucleic acid sequence with a sample suspected of containing telomerase, in the presence of a test compound, under conditions such that if telomerase is present in the sample, the nucleic acid sequence is elongated by telomerase to form an elongated nucleic acid sequence in the absence of the test compound, and

detecting the presence or absence of the elongated nucleic acid sequence.

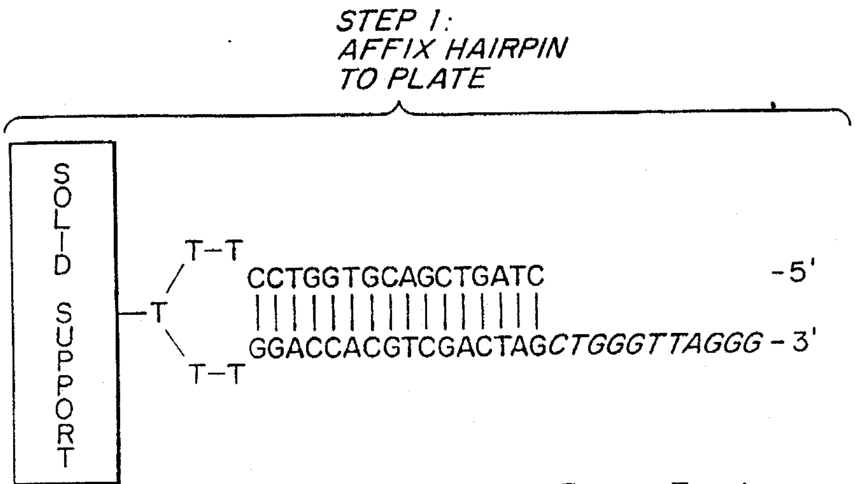
STEP 1:
AFFIX HAIRPIN
TO PLATE
SOLID SUPPORT
T–T
T
T–T
CCTGGTGCAGCTGATC -5'
GGACCACGTCGACTAGCTGGGTTAGGG - 3'

FIG. 1A

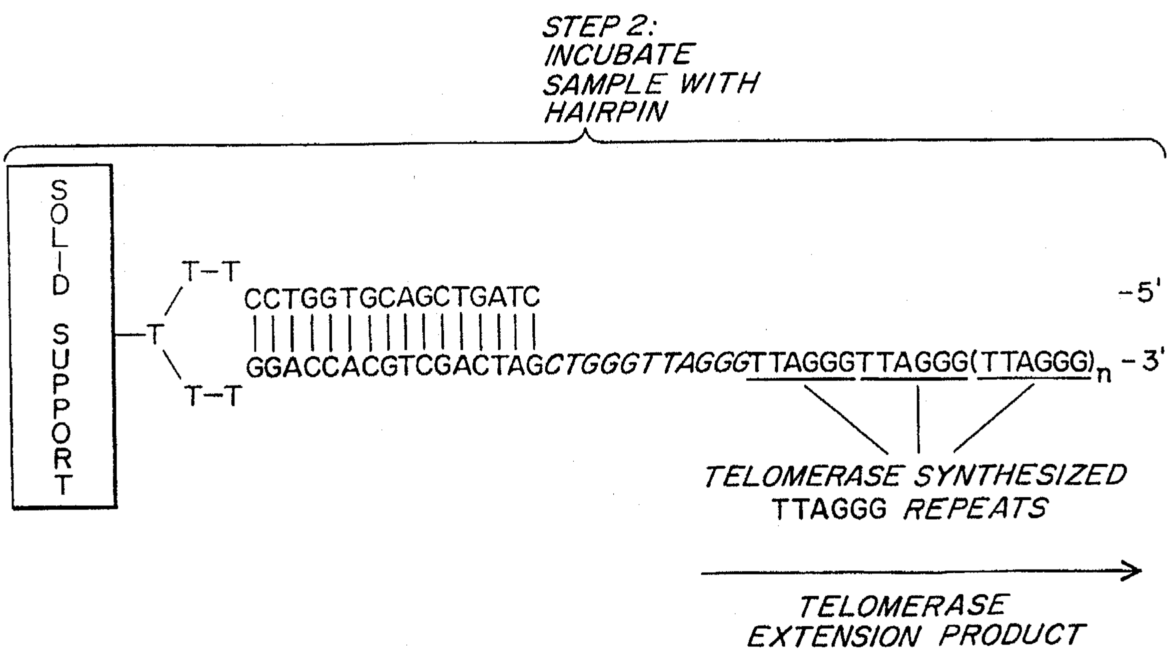
STEP 2:
INCUBATE
SAMPLE WITH
HAIRPIN
SOLID SUPPORT
T–T
T
T–T
CCTGGTGCAGCTGATC -5'
GGACCACGTCGACTAGCTGGGTTAGGGTTAGGGTTAGGG(TTAGGG)n - 3'
TELOMERASE SYNTHESIZED
TTAGGG REPEATS
TELOMERASE
EXTENSION PRODUCT

FIG. 1B

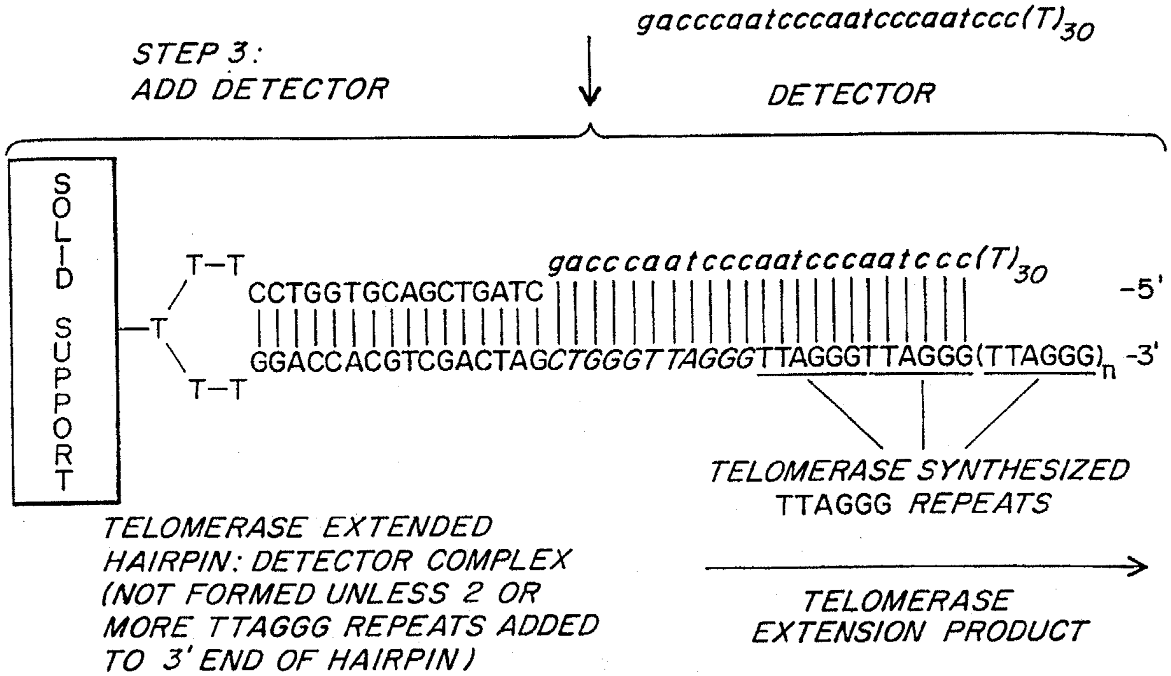
STEP 3:
ADD DETECTOR
gacccaatcccaatcccaatccc(T)30
DETECTOR
SOLID SUPPORT
T—T
T
T—T
CCTGGTGCAGCTGATC gacccaatcccaatcccaatccc(T)30 -5'
GGACCACGTCGACTAGCTGGGTTAGGGTTAGGGTTAGGG(TTAGGG)n -3'
TELOMERASE SYNTHESIZED
TTAGGG REPEATS
TELOMERASE EXTENDED
HAIRPIN: DETECTOR COMPLEX
(NOT FORMED UNLESS 2 OR
MORE TTAGGG REPEATS ADDED
TO 3' END OF HAIRPIN)
TELOMERASE
EXTENSION PRODUCT

FIG. 1C

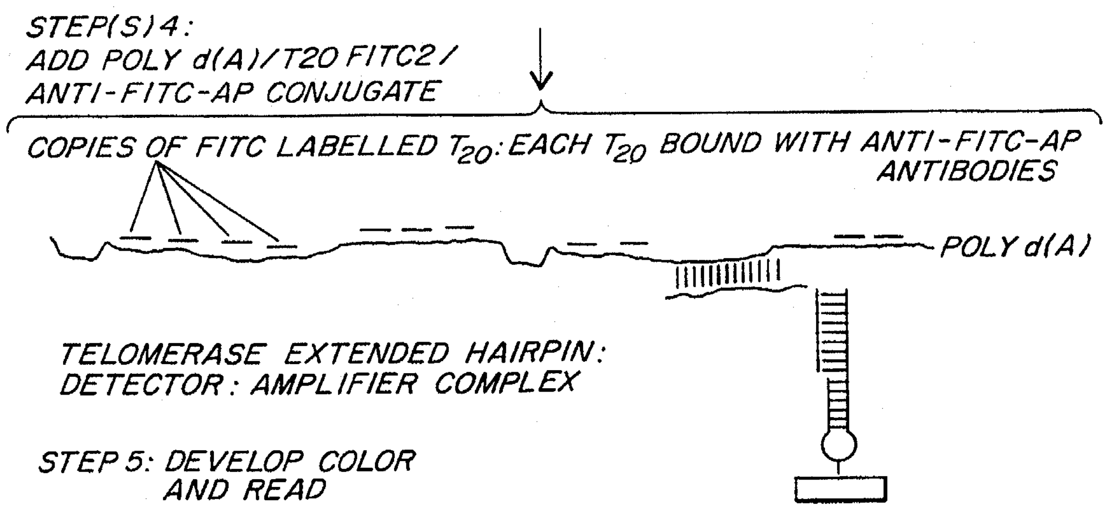
STEP(S) 4:
ADD POLY d(A)/T20 FITC2/
ANTI-FITC-AP CONJUGATE
COPIES OF FITC LABELLED T20: EACH T20 BOUND WITH ANTI-FITC-AP
ANTIBODIES
POLY d(A)
TELOMERASE EXTENDED HAIRPIN:
DETECTOR: AMPLIFIER COMPLEX
STEP 5: DEVELOP COLOR
AND READ

FIG. 1D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03725

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34; C12N 9/12; C07H 21/04
US CL :435/6, 91.1, 194; 536/23.1, 24.3, 24.31; 935/77,78
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 194; 536/23.1, 24.3, 24.31; 935/77,78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS: telomerase, extension

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,489,508 A (WEST et al.) 06 February 1996, claims 1, columns 44-45; column 8, line 66-column 9, line 20.	1, 5
Y	US 4,734,363 A (DATTAGUPTA et al.) 29 March 1988, column 1, lines 36-43, Figure.	1-5
Y	EP 0 297 379 A3 (MOLECULAR DIAGNOSTICS, INC.) 04 January 1989, column 2, line 47-column 3, line 12.	1-5
Y	MANIATIS et al. 'Synthesis And Cloning of cDNA' In: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, pages 214-215, see entire document.	1-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 JUNE 1998	09 JUL 1998

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